Journal of
Applied Crystallography

ISSN 0021-8898

Received 10 December 2002
Accepted 22 January 2003

A procedure for setting up high-throughput nanolitre crystallization experiments. II. Crystallization results

J. Brown,[a] T. S. Walter,[a] L. Carter,[a] N. G. A. Abrescia,[a] A. R. Aricescu,[a] T. D. Batuwangala,[a] L. E. Bird,[a] N. Brown,[b] P. P. Chamberlain,[a] S. J. Davis,[c] E. Dubinina,[b] J. Endicott,[b] J. A. Fennelly,[c] R. J. C. Gilbert,[a] M. Harkiolaki,[d] W.-C. Hon,[a] F. Kimberley,[d] C. A. Love,[a] E. J. Mancini,[a] R. Manso-Sancho,[c] C. E. Nichols,[a] R. A. Robinson,[a] G. C. Sutton,[a] N. Schueller,[b] M. C. Sleeman,[c] G. B. Stewart-Jones,[a] M. Vuong,[c] J. Welburn,[b] Z. Zhang,[e] D. K. Stammers,[a] R. J. Owens,[a] E. Y. Jones,[a] K. Harlos[a] and D. I. Stuart[a]*

[a]Oxford Protein Production Facility and The Division of Structural Biology, The Henry Wellcome Building of Genomic Medicine, Roosevelt Drive, Headington, Oxford OX3 7BN, UK, [b]Laboratory of Molecular Biophysics, Department of Biochemistry, University of Oxford, The Rex Richards Building, South Parks Road, Oxford OX1 3QU, UK, [c]Nuffield Department of Clinical Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, UK, [d]The Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DS, UK, and [e]The Dyson Perrins Laboratory, Oxford Centre for Molecular Sciences, University of Oxford, South Parks Road, Oxford OX1 3QY, UK. Correspondence e-mail: enquiries@strubi.ox.ac.uk

An initial tranche of results from day-to-day use of a robotic system for setting up 100 nl-scale vapour-diffusion sitting-drop protein crystallizations has been surveyed. The database of over 50 unrelated samples represents a snapshot of projects currently at the stage of crystallization trials in Oxford research groups and as such encompasses a broad range of proteins. The results indicate that the nanolitre-scale methodology consistently identifies more crystallization conditions than traditional hand-pipetting-style methods; however, in a number of cases successful scale-up is then problematic. Crystals grown in the initial 100 nl-scale drops have in the majority of cases allowed useful characterization of X-ray diffraction, either in-house or at synchrotron beamlines. For a significant number of projects, full X-ray diffraction data sets have been collected to 3 Å resolution or better (either in-house or at the synchrotron) from crystals grown at the 100 nl scale. To date, five structures have been determined by molecular replacement directly from such data and a further three from scale-up of conditions established at the nanolitre scale.

1. Introduction

The application of nanolitre liquid dispensing technologies to protein crystallization trials offers the opportunity to reduce by one or more orders of magnitude the amount of protein required for initial screening experiments (Santarsiero *et al.*, 2002). Much of the impetus for the implementation of such technology has been generated by its utility for high-throughput structural genomic pipelines. However, the significant reduction in sample quantity coupled to a convenient and reproducible methodology is highly relevant to classical protein crystallographic research projects.

In the accompanying paper (Walter *et al.*, 2003) the modification of commercially available equipment and the design of a protocol for high-throughput nanolitre-scale crystallization experiments is reported. This methodology has already been applied to a broad range of proteins drawn from on-going local crystallographic research projects. The experiences culled from the first few months of operation on an essentially random set of over 50 target proteins and some 1200 crystallization plates guided the development of the protocols reported in the accompanying paper (Walter *et al.*, 2003). They provide encouraging evidence of the general applicability and advantages of automation and miniaturization for crystallization screening and, indeed, the growth of data-collection-quality crystals.

2. Materials and methods

2.1. Range of protein samples

The survey is based on results from 51 proteins. No selection criteria were applied to the sample proteins other than that they were produced for crystallization trials by the authors as part of *bona fide* structural projects in local research groups. As a result, the protein types span various classes of bacterial, fungal and mammalian enzymes, intra-

cellular and extracellular proteins including the extracellular regions of cell surface receptors, structural and non-structural viral proteins. Samples have been variously produced in *E. coli*, insect and mammalian expression systems, generally as soluble proteins, but several examples of refolded proteins are also included. Molecular weights range from 7 to 300 kDa with several samples comprising protein–protein or protein–small-molecule (inhibitor/substrate/cofactor) complexes. The ratio of freshly purified to frozen samples was approximately 3:2, with most having purities in the 95–100% range. Statistics do not allow a useful quantitative correlation of crystallization results with sub-categories of sample type or quality.

2.2. Crystallization-screen reagents

The vast majority of the samples were subjected to a standard survey comprising 5 × 96 crystallization conditions assembled from commercially available kits: Crystal Screen, Crystal Screen 2, PEG/Ion Screen, Grid Screen PEG 6000, Grid Screen Ammonium Sulfate, Natrix, Crystal Screen Cryo, Grid Screen PEG/LiCl, Grid Screen Sodium Chloride, Grid Screen MPD and Quik Screen from Hampton Research (CA, USA), and Emerald BioStructures Wizard Screens I and II from deCODE Genetics (WA, USA).

2.3. Crystallization-screen methodologies

Crystallization trials were conducted by the individual users following training on use of the robotic equipment and advice on the current protocols. Although the majority of the samples were treated identically over the period surveyed, some refinement of protocols did occur in response to the experience gained, most notably a switch from pipetting the reservoir drop first to pipetting the protein drop first. Typically, protein samples were at 5–15 mg ml^{-1} (somewhat lower concentrations were used for some of the largest molecular weight samples and substantially higher concentrations for a few of the very low molecular weight proteins). Set up of the 96-well crystallization trials was always carried out at room temperature (293–295 K); however, some users then transferred their trials to incubate at 278 or 288 K.

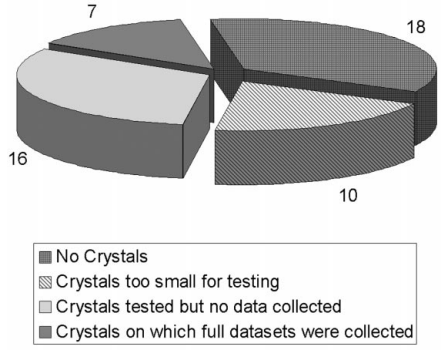

Figure 1
Summary of direct results from nanolitre crystallization trials. No results from subsequent scaling-up are included. The numbers of proteins making up each segment are shown.

A detailed description of the equipment and protocol design is given in the accompanying paper (Walter *et al.*, 2003). Briefly, sitting-drop vapour-diffusion experiments were set up in flat-bottom-platform 96-well crystallization plates (Greiner, Bio-One Ltd, Stonehouse, UK). 96 reservoir solutions (each of 95 µl) were pipetted simultaneously from pre-formatted 96-well master blocks using a Robbins Hydra-96 microdispenser (Apogent Discoveries, Wilmslow, UK). 100 nl:100 nl drops of protein solution and reservoir solution were dispensed (one plate at a time) using a Cartesian Technologies Microsys MIC4000 (Genomic Solutions, Huntingdon, UK), with adaptations as detailed in the accompanying paper by Walter *et al.* (2003). Typically, protein drops were set up first using a single tip in line-dispense mode. Reservoir drops were then added using all eight tips in single-dispense mode. Crystallization plates were sealed with transparent self-adhesive plastic foils as supplied by Greiner (Viewseal) and inspected manually using standard optical microscopes. Crystals were prepared for characterization and X-ray data collection using well established cryo-crystallographic methods. In-house X-rays generated using an RU-300 rotating-anode generator (Rigaku; operating at 48 kV, 100 mA and fitted with Osmic blue multilayer optics) were detected using MarResearch imaging-plate detectors. Synchrotron radiation was accessed on beamlines at either the UK Synchrotron Radiation Source (SRS; Daresbury) or the European Synchrotron Radiation Facility (ESRF; Grenoble, France).

3. Results and discussion

3.1. Success rate, crystal quality and utility

Statistics on the outcome of nanolitre-scale crystallization screens for 51 protein samples are presented in Fig. 1. Of the 51, 18 yielded no crystals in the nanolitre-scale screens (some 'objects' were tested in-house for diffraction but it remains inconclusive whether these were crystalline). None of these 18 samples was reported to have crystallized using standard hand-pipetting methods. Of the 33 samples which yielded identifiable crystals in the nanolitre-scale screen, 23 were considered to be of sufficient size to be tested directly for diffraction. Scale-up of conditions from the nanolitre-scale to standard (hand-pipetted) crystallization drops proved problematic for a significant number of samples (see below) but of the ten projects for which nanolitre-scale crystals were considered too small to test for diffraction, three scaled up successfully, providing crystals suitable for full X-ray data collection.

Of the 23 projects with samples for which nanolitre-scale crystals were tested directly, the majority have been able to make significant progress (four projects report currently insurmountable problems with scale-up and/or optimization of crystal growth). Full X-ray diffraction data set collection from nanolitre-scale crystals has been considered to be practical/useful for three projects in-house and five projects at the synchrotron. Of the three collected in-house, all diffracted to maximum Bragg spacings of 3 Å or better. In one case, a

Table 1
Data-scaling statistics for the IGF2R-Dom11 crystal (see Fig. 2).

Values in parentheses correspond to the highest resolution shell (2.59–2.50 Å).

Space group	$P3_121$
Resolution range (Å)	20–2.5
Wavelength (Å)	1.54
Measurements	18482
Unique reflections	4883
Completeness (%)	95.7 (99.8)
$I/\sigma(I)$	13.9 (5.0)
$R_{\rm merge} = \sum \lvert I - \langle I \rangle \rvert / \sum \langle I \rangle$ (%)	11.7 (36.1)

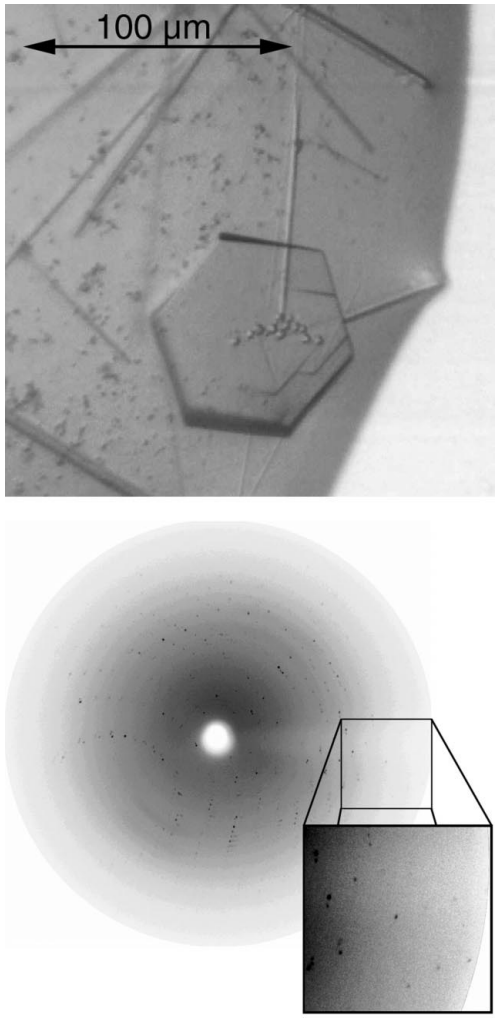

Figure 2
(*a*) Crystal grown from a 100 nl drop of domain 11 of the insulin-like growth factor 2 receptor (IGF2R-Dom11; Brown *et al.*, 2002) plus 100 nl reservoir solution as part of a screen for complexes with a putative small-molecule inhibitor. This is an example of a crystal form discovered in nanolitre-scale trials which had not previously been seen in microlitre-scale trials. (*b*) Representative in-house diffraction image from the crystal shown in (*a*). Data-scaling statistics are given in Table 1.

crystal grown from a 100 nl drop of 14 mg ml^{-1} protein solution plus 100 nl reservoir yielded an in-house data set to 2.5 Å resolution (further details are given in Fig. 2 and data-scaling statistics are given in Table 1).

Although the number of samples investigated to date is too small to justify a statistical analysis of the results, of the proteins for which potentially useful X-ray diffraction data have been collected, about 30% were produced in eukaryotic expression systems and about 70% were proteins of eukaryotic or viral origin. The diffraction-quality crystals include protein–oligonucleotide and protein–inhibitor complexes and most are multi-domain. Overall, we see no indication that the use of small drops introduces any bias into the outcome of the experiments.

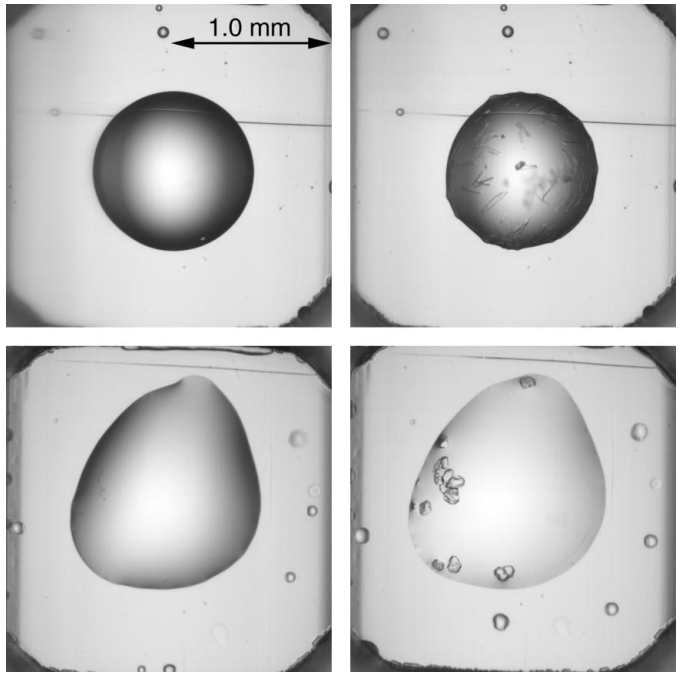

Figure 3
Time-lapse photographs showing the formation of glucose isomerase crystals. Images (*a*) and (*b*) show crystals grown in 30% polyethylene glycol 400, 0.1 *M* Na-HEPES, pH 7.5, 0.2 *M* magnesium chloride (Hampton research Crystal Screen solution #23), and photographed at $t = 0$ h and $t = 40$ h, respectively. Images (*c*) and (*d*) show crystals grown in 30% 2-propanol, 0.1 *M* Tris-HCl, pH 8.5, 0.2 *M* ammonium acetate (Hampton research Crystal Screen solution #19), and again photographed at $t = 0$ h and $t = 40$ h, respectively. Images were taken by the OASIS 1700 image-acquisition system (Veeco, Cambridge, UK).

3.2. Nanolitre scale versus standard drop-size screens

Not all of the 51 sample proteins have been screened for crystallization conditions using standard hand-pipetting techniques (the nanolitre technology has become the method of choice for many of the authors), but where comparisons can been drawn, no protein fails to crystallize in the nanolitre-scale drops which has succeeded in the standard drops (although for one project fewer conditions yielded crystals at the nanolitre scale). For a significant number of samples, the nanolitre scale screen identified new crystallization conditions and for at least five of the proteins, crystals could only been grown by this method, a finding echoing the report of Bodenstaff *et al.* (2002). At present, problems are frequently encountered in scale-up and crystal optimization. One factor that may contribute to this is the generation, due to mixing in the Cartesian tips, of a 'protein gradient' across the plate, as described in the accompanying paper (Walter *et al.*, 2003).

Until this problem is solved, it may be necessary to take this into consideration during optimization and scale-up. In our experience, refinement of crystallization conditions initially identified using nanolitre-scale technology is generally best done by re-screening with the Cartesian robot and a customized screen.

3.3. Rate of crystal nucleation and growth

Crystal growth times in nanolitre-scale drops still show substantial variation from sample to sample (example time-lapse images are shown in Fig. 3). One author reports that crystals are first visible within 10 min of crystallization-screen set up, growing to maximum size over 2–5 days. Others report crystals appearing between 12 h and 3 weeks of screen set up. Kuhn *et al.* (2002) have reported a trend towards more rapid crystal growth in nanolitre-scale trials and, although similar behaviour has been noted in this survey, the experiences reported for several of the current samples indicates that this is by no means generic. Several samples do, however, reveal increased problems with multiple nucleation sites in microlitre-scale drops compared with nanolitre-scale drops, a feature which has proved problematic for scale-up attempts.

3.4. Technical glitches

Day-to-day use of the nanolitre technology uncovered several technical glitches. The first encountered was a frequent tendency for drops to migrate to the edges of the flat wells, making visualization and imaging difficult. This movement was most frequent when reservoir drops were pipetted first and was rarely seen when protein was dispensed first. There was no direct correlation between reservoir constituents and drop migration, and there was no evidence of a static charge on the plate being responsible. As a consequence of migration, there were occasions when protein and reservoir drops failed to mix, giving separate drops in the well. As a result of these observations, the experimental procedure was altered so that protein is dispensed before reservoir; now drop migration rarely occurs and if it does, the drops have already mixed.

Some users reported drop evaporation despite the presence of the Viewseal foil on the plate. As noted in the accompanying paper (Walter *et al.*, 2003), these foils contain an encapsulated sealant, which is released only where the foil is pressed against a solid support. Since evaporation problems were not widespread, they probably reflected poor sealing; it should be noted that pressing using a firm surface such as a fingernail produces a much better seal than simply pressing with ones thumb.

As part of the experimental procedure, there are several wash steps where the ceramic tips are cleaned and dried under a vacuum. Despite this, tips can eventually become blocked, presumably because of an internal build up of salts and other components of the reservoir solutions. Blockages can be precluded by regularly detaching and immersing the tips in a mild detergent solution and cleaning in an ultrasonic water bath for 5 min.

4. Conclusions

We have demonstrated that the protocol described is capable of producing useful crystals at a high success rate. Further developments will include a protocol for the systematic use of additive screens and automated optimization of initial conditions. In addition, we would like to explore the use of both smaller (for screening) and larger (for optimization) drops.

Reduction of the protein and reservoir solution volumes required for screening crystallization conditions, coupled with increases in accuracy, efficiency and reproducibility, constitute the obvious benefits of an automated high-throughput procedure. The observation that nanolitre-scale procedures can lead directly to the growth of diffraction-quality crystals yielding full data sets is an enormous bonus. We have found that the automation described in this paper can be applied in classical protein crystallography laboratories and it is easy to envisage similar procedures replacing traditional hand-pipetting methods for crystallization screening and optimization. Indeed, the method has been embraced enthusiastically by the crystallographic community in Oxford, such that it is already becoming the preferred *modus operandi* for many workers. Although there is a significant capital cost with respect to the equipment, experience to date indicates that for an active laboratory, especially one routinely producing proteins in eukaryotic expression systems, this cost is likely to be recouped relatively quickly. Furthermore, where the level of protein production is very low or cofactors or ligands are very expensive, small drops may allow crystallization experiments to be contemplated which would otherwise be prohibitively expensive.

The Oxford Protein Production Facility is a Medical Research Council (MRC) funded pilot project for the UK and is part of the Structural Proteomics IN Europe (SPINE) consortium (European Commission Grant No. QLG2-CT-2002-00988). Protein samples were drawn from on-going research projects funded by the Biotechnology and Biological Science Research Council, Cancer Research UK, the European Commission, The Human Frontier Science Program, MRC, The Wellcome Trust and Arrow Therapeutics. We thank the staff at SRS Daresbury, ESRF Grenoble and MRC France for assistance with data collection.

References

Bodenstaff, E. R., Hoedemaeker, F. J., Kuil, M. E., De Vrind, H. P. & Abrahams, J. P. (2002). *Acta Cryst.* D**58**, 1901–1906.

Brown, J., Esnouf, R. M., Jones, M. A., Linnell, J., Harlos, K., Hassan, A. B. & Jones, E. Y. (2002). *EMBO J.* **21**, 1054–1062.

Kuhn, P., Wilson, K., Patch, M. G. & Stevens, R. C. (2002). *Curr. Opin. Chem. Biol.* **6**, 704–710.

Santarsiero, B. D., Yegian, D. T., Lee, C. C., Spraggon, G., Gu, J., Scheibe, D., Uber, D. C., Cornell, E. W., Nordmeyer, R. A., Kolbe, W. F., Jin, J., Jones, A. L., Jaklevic, J. M., Schultz, P. G. & Stevens, R. C. (2002). *J. Appl. Cryst.* **35**, 278–281.

Walter, T. S., Diprose, J., Brown, J., Pickford, M., Owens, R. J., Stuart, D. I. & Harlos, K. (2003). *J. Appl. Cryst.* **36**, 308–314.